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Ligation of CD40 with soluble CD40 ligand reverses anti-immunoglobulin-mediated negative signalling in murine B lymphoma cell lines but not in immature B cells from neonatal mice

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SUMMARY

Ligation of surface immunoglobulin (sIg) on certain murine B-lymphoma lines has been shown to initiate a programme leading to growth arrest and death of the cells by apoptosis. The cell lines WEHI 231 and CH33 which respond in this way to receptor cross-linking have phenotypic characteristics resembling those of immature normal B cells, and their responses have been taken to model those responsible for clonal deletion or anergy. Cross-linking of sIg on normal neonatal B cells has also been shown to inhibit their responsiveness to polyclonal activators. We have examined the ability of various co-stimuli to modify the response of growth-inhibitable B lymphoma lines to sIg cross-linking. Our findings indicate that cell–cell contact between cells of the WEHI 231 or CH33 lines and activated T cells rescues these cells from growth arrest and apoptosis. Cell-free supernatants from some T-cell lines were also protective although recombinant IL-4 had no effect. Analysis of the most effective signals and timing for inducing this protection suggested that it might, in part, be mediated by CD40 ligand (CD40L) expressed on or secreted by activated T cells. Using a soluble recombinant CD40L-CD8 fusion protein we have now shown that co-ligation of CD40 is sufficient to rescue WEHI 231 and CH33 cells from anti-Ig-induced apoptosis. In contrast, the inhibitory effect of anti-Ig antibodies on the lipopolysaccharide (LPS)-driven proliferation of neonatal B cells was not relieved by co-ligation of CD40 with CD40L. These findings bring into question the usefulness of 'immature' B-cell lines as models for tolerance induction.

INTRODUCTION

The generation of antigen-specific immunity is characterized by the proliferation of those clones of lymphocytes possessing cell-surface receptors capable of binding the stimulating antigen. Cross-linking of clonotypic receptors on both T and B lymphocytes initiates a cascade of events that result in the exit of the cells from G_0 and entry into active cycle. Once activated, cells may pass through several or many division cycles before differentiating to effector or memory cell phenotypes. Investigation of the mechanisms that underlie these events has mostly been confined to defining the molecules required to facilitate entry into cycle, maintain cellular proliferation or induce one or other effector functions.[1,2]

In mature resting cells the cross-linking of sIg receptors by multivalent antigen leads to cellular activation by mechanisms that involve the phosphorylation on tyrosine, serine or threonine residues of a variety of cellular substrates,[3,4] changes in inositol lipid metabolism[5] and elevation of intracellular calcium levels.[6] In turn these events, either directly or via the activation of one or more enzymic cascades, lead to transcriptional changes allowing the synthesis of new protein molecules and modifying the expression of others.[7] Thus B-cell receptor occupancy is followed by an upregulation of surface expression of class II MHC molecules,[8] serving to enhance the ability of the cell to present processed antigen to T cells. The developing T:B interaction has been shown to result in the reciprocal stimulation of both participants in a process which involves cell-adhesion molecules[9,10] and includes the participation of other receptor/ligand pairs[11,12] and cytokines. Recent work suggests that the interactions between CD28/CTLA-4 and CD40L on T cells and their counter structures, B7-1/B7-2 and CD40 on B cells play a particularly crucial role in driving T- and B-cells responses, including isotype switching and somatic mutation events which are required for vigorous and effective immunity.[13,14]

Tolerance of self-antigen by the immune system, however, requires a different response in which self reactive B cells are either eliminated or rendered unresponsive to antigen. Under certain circumstances the ligation of antigen-specific receptors has been shown to produce inhibitory rather than stimulatory

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effects. Thus T cells presented with antigen delivered to B lymphocytes in monomeric form became anergic, whilst the same antigen delivered to B cells in multivalent form (and hence capable of cross-linking sIg) produced a vigorous and specific T-cell response.[15]

Immature, $sIgM^+ IgD^-$ B cells from bone marrow or neonatal spleen have been shown to be exquisitely sensitive to tolerance induction following sIg ligation with antigen.[16,17] Similarly, although adult B cells normally respond to anti-immunoglobulin-induced sIg cross-linking by proliferating, neonatal B cells are refractory to this stimulus[18] and have been shown to be inhibited from responding to the B-cell mitogen LPS when co-cultured with anti-immunoglobulin.[19,20] There is also a need to eliminate those B cells with self-reactive receptors that may arise as a result of the somatic mutation events that occur during memory generation, suggesting that mature cells with the characteristics of germinal centre cells may also be tolerance susceptible.

Evidence from transgenic systems has demonstrated that several mechanisms may underpin tolerance induction. Self-antigens that extensively cross-link antigen receptors were shown to cause the elimination of both immature self-reactive B cells in the bone marrow[21] and mature B cells in the periphery.[22] Other antigens that cause less extensive cross-linking did not cause deletion of the B-cell clones that recognized them but did produce unresponsiveness by rendering the B cells anergic[23] or preventing their access to lymphoid follicles.[24] These experiments also suggest that the clonal deletion induced in immature B cells by receptor cross-linking in the absence of a second signal derived from T cells may occur as a result of the engagement of a cell death programme that results in apoptosis, by a similar process to that which is responsible for eliminating self-reactive T cells in the thymus.[25] Recently, direct evidence has been obtained to show that both immature[26] and mature[27] B cells can be induced to undergo sIg-mediated apoptosis. Several B-lymphoma lines that phenotypically resemble immature B cells have been used as a models for deletional tolerance and the delivery of negative signals via sIg. Cells of the murine WEHI 231[28] and CH33[29] lines show growth arrest and undergo apoptosis[30] when cultured with cross-linking anti-immunoglobulin antibodies, and cells of the human Epstein–Barr virus (EBV)-transformed Ramos line behave in a similar fashion.[31]

It has long been known that B-cell tolerance is more easily induced in the absence of T cells[16,32] and using the aforementioned cell lines it has been shown that T-cell-derived signals including interleukin-4 (IL-4) may prevent the effects of sIg ligation and rescue the cells from apoptosis.[33] Ligation of CD20 or CD40 on Ramos cells has also been shown to reduce the number of cells undergoing apoptosis as a consequence of sIg ligation. Confirmation of a role for CD40 in protecting B cells from the induction of programmed cell death has also been obtained in experiments in which sIg-mediated apoptosis of WEHI 231 cells was abrogated by association of CD40 on their surface with CD40L-transfected myeloma cells or by reaction of human CD40 transfected into WEHI 231 cells with a human CD40-specific monoclonal antibody (mAb).[34] To further examine the role of T-cell-derived signals in modulating negative signalling in B cells we have employed both lymphoma cell lines and neonatal B cells which are receptive to such signals, and investigated the effects of activated T cells and their products on the induction of growth arrest and/or apoptosis induced by sIg cross-linking. Our results indicate that the signals that rescue these cells from negative signalling are distinct and suggest that, contrary to the widely held view, transformed lines do not provide good models for clonal deletion of immature B cells from neonatal mice.

MATERIALS AND METHODS

Cell lines

Cells of the WEHI 231 and CH33 lines were maintained at 2–10×10^5 cells/ml by twice-weekly subculture in RPMI-1640 supplemented as described below. Transfected J558 cells secreting the CD40L–CD8 fusion protein[35] (a gift from Dr P. Lane, Basel, Switzerland) were maintained in supplemented RPMI-1640 containing 2 mg/ml Geneticin (Life Technologies, Paisley, UK). For production of CD40L containing supernatants the cells were cultured at 10^6 per ml for 24 hr without selection agent, the cell-free supernatants harvested, filtered (0·22 μm) and stored at −70° until used.

T-cell lines

The CD4-positive CBOVA and BA5-6E69$^-$ (BA5) T-cell lines were a generous gift from Dr D. B. Thomas [National Institute for Medical Research (NIMR), Mill Hill]. The CBOVA line is specific for ovalbumin in the context of $I\text{-}A^k$ and the BA5 line[36] for influenza haemagglutinin in the context of $I\text{-}A^d$. The lines were maintained by stimulating with the relevant antigen (ovalbumin, 100 μg/ml or inactivated X31 virus, 100 HAU/ml) in the presence of irradiated spleen cells bearing the appropriate major histocompatibility complex (MHC) haplotype for 3 days with the addition of interleukin-2 (IL-2) containing supernatant (10% v/v) for a further 7 days. For the production of supernatants, cells collected at the end of the above culture period were harvested, washed and recultured at 2×10^6/ml with antigen in the presence of feeder cells for 20 hr. Cell-free supernatants were then collected, filtered (0·22 μm) and stored at −20° until used.

Preparation of resting and activated T cells

Spleen cells from BALB/c mice were depleted of B lymphocytes by treatment with mAb LR-1[37] and complement. Spleen cells ($1–2 \times 10^7$ per ml) were incubated with mAb LR-1 (1 : 1000 in RPMI-1640) for 45 min on ice, washed and incubated with guinea pig serum (1 : 10) for a further 45 min at 37°. The resulting cell suspension (>90% Thy-1 positive) was washed twice in RPMI-1640, resuspended in RPMI-1640 supplemented with fetal calf serum (FCS) (5% v/v), penicillin (50 U/ml), streptomycin (50 μg/ml), L-glutamine (2 mM), sodium pyruvate (1 mM) and 2-mercaptoethanol (5×10^{-5} M) and cultured at 2×10^6 cells per ml for 20 hr at 37° in medium alone (resting T cells), or in medium containing Concanavalin A (Con A) (2 μg/ml) or hamster anti-mouse CD3 mAb 145–2C11 (0·1% v/v hybridoma supernatant) to produce activated T cells. After culture, resting and activated cells were washed in medium containing α-methyl mannoside (100 μg/ml) and either irradiated (20 Gy) or treated with mitomycin C (50 μg/ml) for 45 min at 37° to block proliferation. After treatment the cells were washed twice more prior to culture with WEHI 231 cells as described below.

Cell-proliferation assays

Growth inhibition of WEHI 231 and CH33 cells was assessed in a proliferation assay as described previously.[37] Briefly, WEHI 231 or CH33 cells were cultured in 96-well plates at 10^4 cells per well in the presence of various concentrations of rat anti-mouse κ chain mAb 187.1 for 48 hr and proliferation assessed by pulsing with [^{3}H]thymidine (0·5 μCi/well) for the last 4 hr of culture. Results, expressed as c.p.m. incorporated, or as percentage inhibition compared with cultures without anti-κ antibody, are based on at least triplicate cultures for each point. To examine the effects of T cells, T-cell supernatants and CD40L on growth inhibition, cultures set up as above were supplemented with resting or activated T cells, the supernatants of T-cell lines or CD40L–CD8 fusion protein containing supernatants with or without anti-CD8 mAb as indicated in the text. In some experiments WEHI 231 cells were also cultured with anti-κ antibody in the presence of purified recombinant murine IL-4 (Genzyme, Cambridge, MA).

Neonatal B cells were enriched from spleen cell suspensions prepared from 7-day-old mice as follows: Spleen cells were depleted of T cells by incubation with a mixture of anti-Thy1.2 mAbs 70 and 97 (1 : 1000) and guinea pig complement, and of adherent cells by culture in tissue culture-treated petri dishes at 4×10^6 per ml for 2 hr at 37°. B cells were further enriched by centrifugation of the remaining cells over 67% v/v Percoll. Cells collected from the interface were washed three times before use and were routinely >40% sIg positive.

Antibodies

The following monoclonal antibodies were used in this study: clones 70 and 97 (anti-mouse Thy1.2, a gift from Dr D. B. Thomas); 187.1[38] (rat anti-mouse κ chain); b7.6[39] (rat anti-mouse μ chain); 53.6.72[40] (rat anti-mouse CD8); 145-2C11[41] (hamster anti-mouse CD3); LR-1 (rat anti-mouse B cells). The antibodies were used in the form of tissue culture supernatants of the appropriate hybridoma cell line, partially purified by precipitation with 45% saturated NH_4SO_4 or purified by chromatography on protein G agarose.

Measurement of DNA fragmentation

The induction of apoptosis in WEHI 231 cells was assessed by a modification of the method described by Hasbold & Klaus.[30] Briefly, WEHI 231 cells cultured (5×10^5/ml) with [^{3}H]thymidine (0·5 μCi/ml) for 4 hr were washed and plated in 24-well plates (2×10^5/well) in the presence or absence of anti-κ mAb (3 μg/ml) with or without CD40L–CD8 supernatant and anti-CD8 mAb as indicated in a final volume of 2 ml. After 15 hr the cells were harvested, washed with cold phosphate-buffered saline (PBS) and lysed with 0·5% v/v NP40 or 1% sodium dodecyl sulphate (SDS) in lysis buffer. The samples were centrifuged for 5 min at 10 000 ***g*** and release of labelled material into the supernatants measured by liquid-scintillation spectroscopy. Percentage DNA fragmentation was calculated by comparing the release of label into NP40 lysates of treated or untreated cells with that released by total lysis (SDS) of untreated control cells.

RESULTS

Effect of activated T cells on growth inhibition of WEHI 231 cells

When cultured in the presence of anti-μ or anti-κ antibodies, cells of the WEHI 231 cell line exhibit marked growth inhibition[28,37] and eventually undergo cell death by apoptosis.[30] The effects of T cells on anti-immunoglobulin-mediated growth arrest were examined by the addition of mitomycin-treated, Con A-activated purified mouse T cells to cultures of WEHI 231 cells in the presence or absence of anti-κ chain antibody. The results (Fig. 1) show that the presence of T cells activated with Con A completely reversed the growth inhibition produced by anti-κ at 0·3 μg/ml and partially reversed that produced at 3 μg/ml. A ratio of T cells to WEHI 231 cells of 20 : 1 was more effective in this regard than a ratio of 6 : 1. These findings suggested that a molecule expressed on the surface of activated T cells or secreted by them was capable of interacting with WEHI 231 cells to induce signals counteracting those generated by ligation of sIg.

Effect of T cells depends on their mode of activation

To further investigate the need for T cells to be activated to effectively reverse anti-immunoglobulin-mediated inhibition, WEHI 231 cells were co-cultured with unstimulated T cells or T cells cultured for various times with Con A or mAb anti-CD3.

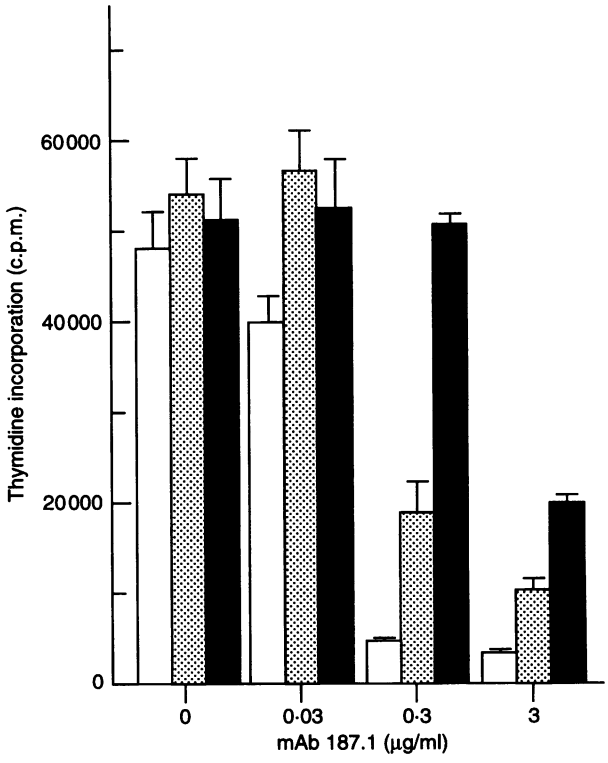

Figure 1. Effect of Con A-activated T cells on anti-immunoglobulin-induced growth inhibition of WEHI 231 cells. BALB/c spleen cells, depleted of B cells by treatment with mAb LR-1 (90% Thy.1 positive) were activated by incubation with Con A (2 μg/ml) for 20 hr, washed and treated with mitomycin C. WEHI 231 cells (10^4 per well) were cultured without activated T cells (□) or with 6×10^4 (▨) or 2×10^5 (■) cells in the presence of various concentrations of rat anti-mouse κ mAb (187.1) as shown. Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hr. Values shown are the means ±SD of triplicate wells. Mitomycin-treated cells (2×10^5) incorporated <1000 c.p.m. when cultured alone.

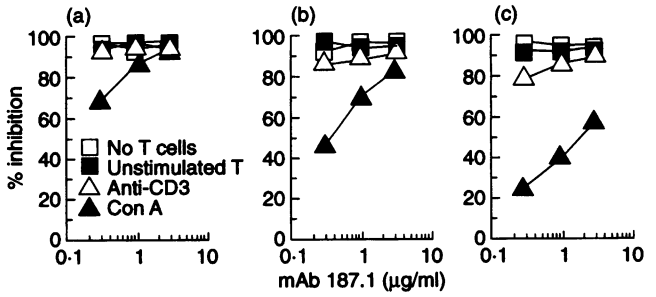

Figure 2. Con A-activated but not resting or anti-CD3-activated T cells reverse anti-immunoglobulin-induced growth arrest of WEHI 231 cells. B-depleted spleen cells activated with Con A or anti-CD3 or incubated in medium for 48 hr as indicated, were irradiated and cultured at 5×10^4 (a), 10^5 (b) or 2×10^5 (c) cells per well with WEHI 231 cells (10^4 per well) in the presence of various concentrations of mAb anti-κ (187.1). Proliferation was assessed by measuring [^{3}H]thymidine uptake at 48 hr. Results are shown as percentage inhibition of [^{3}H]thymidine incorporation compared with cultures without anti-κ and are based on triplicate cultures for each point. Irradiated T cells incorporated <1000 c.p.m. when cultured alone.

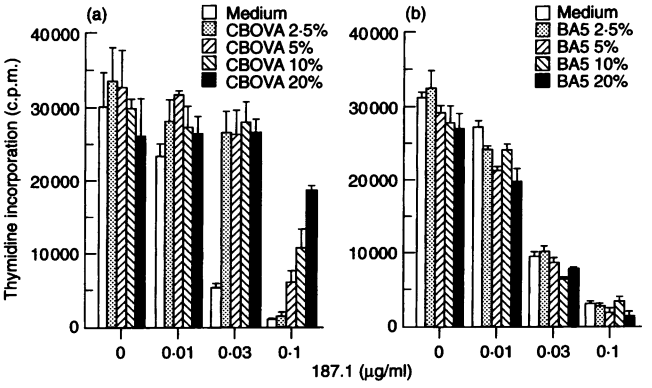

Figure 3. Effect of T-cell supernatants on anti-immunoglobulin-induced growth inhibition of WEHI 231 cells. WEHI 231 cells (10^4 per well) were cultured with anti-κ mAb with various concentrations of supernatants from the CBOVA (a) and BA5 (b) T-cell lines as indicated. Proliferation was assessed at 48 hr. Values shown represent the means ±SD of triplicate cultures.

As can be seen from Fig. 2, unstimulated T cells were completely ineffective in reversing anti-immunoglobulin-induced growth arrest, whilst even low numbers of Con A-activated cells had a marked effect. Interestingly, T cells activated by soluble anti-CD3 were ineffective—although this mode of stimulation did induce T-cell proliferation comparable to that induced by Con A (data now shown). T cells activated with Con A for 16 hr were as effective as cells activated for 48 hr but cells activated for 72 hr were less active (data not shown). Thus cellular activation appears to be required for T cells to exhibit this effect and the mode of activation is important. The ability of T cells to reverse anti-immunoglobulin-mediated growth inhibition is unlikely to be due to contaminating B cells because these comprised less than 5% of the total cell population and because unstimulated cells (which would contain similar numbers of B cells) showed no effect.

Rescue of WEHI 231 cells from growth arrest by soluble T-cell products

Several reports have indicated that cytokines or other soluble products released by T cells are capable of preventing the anti-immunoglobulin-mediated growth inhibition of WEHI 231 cells.[33,34] This suggested that the effects observed with activated T cells might result from the secretion or release of soluble molecules following activation. This possibility was addressed by examination of the effects of cell-free supernatants from two T-cell lines on the proliferation of WEHI 231 cells cultured in the presence or absence of anti-κ antibody. The results (Fig. 3) showed that growth inhibition was effectively reversed in cultures containing supernatant from the CBOVA line but not by supernatants of the BA5 line. Irradiated, activated CBOVA cells also prevented growth arrest but BA5 cells did not. Although we have not phenotyped the CBOVA line it was obtained from CBA mice following conventional immunization with ovalbumin in a protocol that might be expected to generate Th2-type lines. The BA5 line, on the other hand, was derived from BALB/c mice following a natural infection with the X31 influenza virus and is a typical Th1-type line.[36]

CBOVA supernatants were shown to contain IL-4 as judged by their ability to induce anti-IL-4-sensitive MHC class II upregulation on mouse B cells (data not shown). No IL-4 could be detected in supernatants of the BA5 line, but these cells have been shown to secrete interferon-γ (IFN-γ). The data thus suggested a potential role for IL-4 in the prevention of growth arrest.

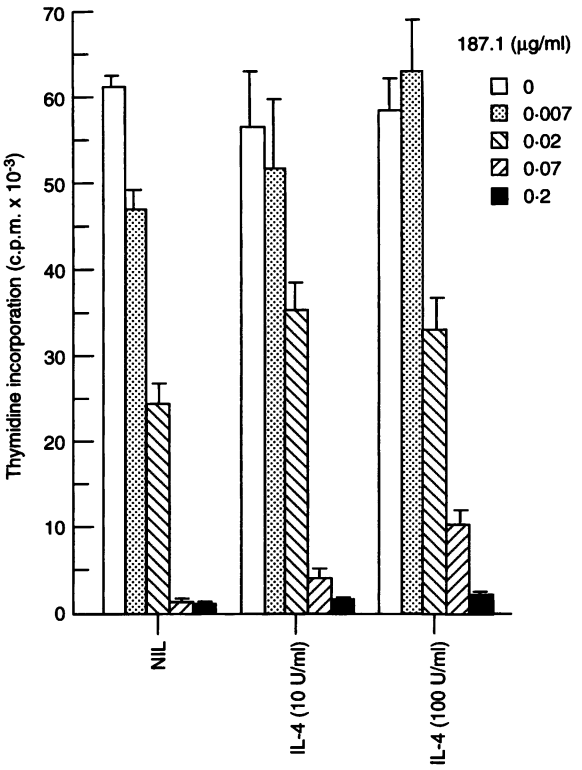

Figure 4. IL-4 does not prevent anti-immunoglobulin-induced growth arrest of WEHI 231 cells. WEHI 231 cells (10^4 per well) were cultured with anti-κ mAb with various concentrations of purified recombinant IL-4 as indicated. Proliferation was assessed at 48 hr. Results shown are the means ±SD of triplicate cultures.

IL-4 does not rescue WEHI 231 cells from growth arrest

For a direct examination of the role of IL-4, WEHI 231 cells were cultured with anti-κ antibody in the presence or absence of recombinant IL-4 (rIL-4). Cells cultured in the presence of the cytokine showed essentially similar inhibitory responses to those cultured in its absence (Fig. 4). This data, which contrasts with the observations made by Scott *et al.*,[33] implied a role for T-cell-derived molecules other than IL-4 in modifying the response of WEHI 231 cells to sIg ligation.

Co-ligation of CD40 with its natural ligand overrides anti-immunoglobulin-induced apoptosis

Recent reports have shown that the expression of the natural ligand for CD40 (CD40L) on T cells is activation dependent and that certain modes of activation, including CD3 ligation, are less effective than others in inducing its upregulation.[42] The inability of CD3-activated T cells to prevent anti-immunoglobulin-induced growth arrest in WEHI 231 cells suggested that cell-surface expression of CD40L on the activated T cells may be required for this effect. To test this directly, the ability of a CD40L–CD8 fusion protein to reverse the growth inhibition of WEHI 231 and CH33 cells by anti-immunoglobulin-antibody was examined. The results (Fig. 5) show that ligation of CD40 with CD40L partially reversed the effects of anti-κ on both cell lines and that the effectiveness of the CD40L was markedly enhanced when the fusion protein was cross-linked by the addition of anti-CD8 mAb. Essentially similar results were obtained when growth arrest was induced with anti-μ antibody (not shown). Cell-free supernatants from untransfected J558 cells had no effect. In time-course experiments this protective effect was apparent even when addition of CD40L was delayed by up to 12 hr after addition of anti-immunoglobulin (data not shown).

The induction of growth arrest in WEHI 231 cells following sIg ligation has been shown to involve the triggering of a programme that leads to cell death by apoptosis[30] with the appearance of fragmented DNA. The effect of CD40L on this process was assessed by determining the extent of DNA fragmentation produced in WEHI 231 cells by sIg ligation in the presence or absence of the CD40L–CD8 fusion protein. Treatment with anti-immunoglobulin-induced marked DNA fragmentation and the extent of this was clearly reduced in the presence of soluble CD40L. This effect of CD40L was dramatically enhanced when the fusion protein was cross-linked by anti-CD8 (Fig. 6).

Ligation of CD40 does not prevent anti-immunoglobulin-mediated inhibition of LPS responses by neonatal B cells

The growth-inhibitory response of WEHI 231 and CH33 cells has been widely exploited as a model for the mechanisms involved in tolerance induction in immature cells.[28,43,44] This is partly supported by observations that the LPS-induced

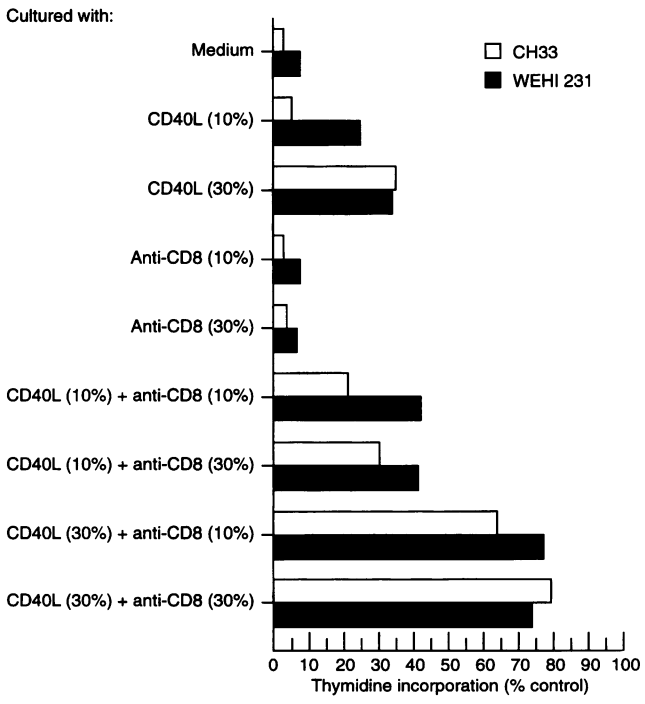

Figure 5. Soluble CD40 ligand rescues WEHI 231 and CH33 cells from anti-immunoglobulin-induced growth arrest. WEHI 231 or CH33 cells (10^4 per well) were cultured in the presence or absence of anti-κ mAb (3 μg/ml) with various concentrations of supernatant containing the CD40L-CD8 fusion protein with and without anti-CD8 supernatant as indicated. Proliferation was assessed at 48 hr. Values shown represent the percentage of [^{3}H]thymidine incorporation in cultures containing anti-κ compared with controls lacking this reagent and are based on triplicate cultures for each point.

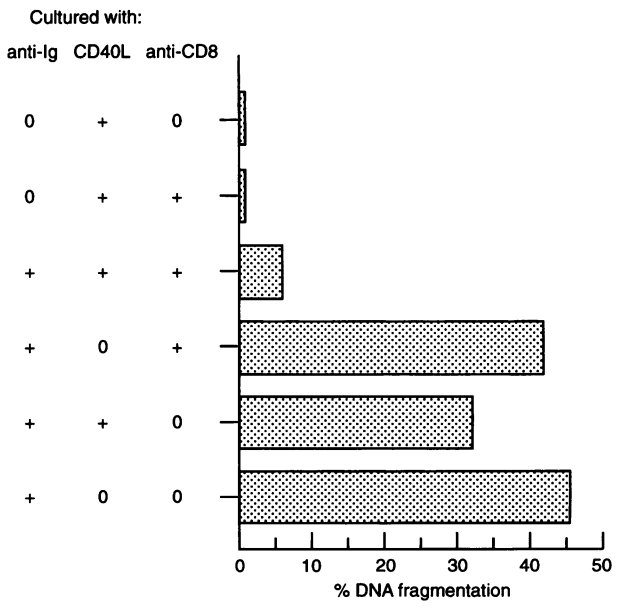

Figure 6. Ligation of CD40 with soluble CD40L rescues WEHI 231 cells from anti-immunoglobulin-induced apoptosis. WEHI 231 cells (5×10^5/ml) labelled with [^{3}H]thymidine for 4 hr were washed and recultured (2×10^5 per well) in the presence of CD40L-CD8 supernatant (25% v/v) and anti-CD8 mAb supernatant (10% v/v) with or without anti-κ mAb as indicated. Cells were harvested after 15 hr and DNA fragmentation assessed by release of labelled material after lysis with NP40 as described in the Materials and Methods. Values shown were determined from triplicate cultures.

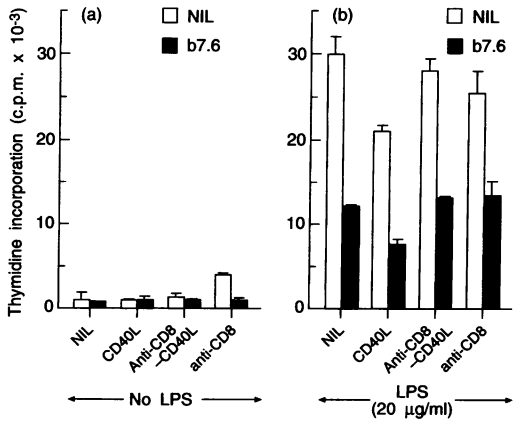

Figure 7. CD40L fails to reverse the anti-immunoglobulin-induced inhibition of LPS responses by neonatal B cells. Spleen cells from 7-day-old mice were enriched for B cells and cultured (2×10^5 per well) for 72 hr with CD40L-CD8 supernatant (25% v/v), anti-CD8 mAb (1 μg/ml), rat anti-mouse μ mAb (b.7.6, 10 μg/ml) with (b) or without (a) LPS (20 μg/ml) as indicated. Proliferation was assessed by pulsing with [^{3}H]thymidine for the last 4 hr of culture. Results shown are means ±SD of triplicate cultures and are representative of four similar experiments.

Table 1. Contrasting effects of anti-immunoglobulin antibody on adult and neonatal B cell responses

Cells cultured with			Thymidine incorporation (c.p.m.)	
CD40L*	LPS†	anti-μ‡	Adult B cells	Neonatal B cells
−	−	−	2099 ± 362	2092 ± 653
+	−	−	23 700 ± 3833	9118 ± 999
−	−	+	4374 ± 531	1978 ± 273
+	−	+	37 958 ± 4879	3727 ± 791
−	+	−	50 677 ± 4657	27 956 ± 1192
+	+	−	95 261 ± 1052	77 528 ± 3930
−	+	+	50 141 ± 1934	16 524 ± 1821
+	+	+	94 528 ± 7155	40 349 ± 2296

Splenic B cells from adult (12-week old) or B-enriched cells from neonatal (6-day old) mice were cultured at 2×10^5 per well for 48 hr. Results shown are the mean ±SD of triplicate cultures.

* CD40L-CD8 s/n (25% v/v) + anti-CD8 mAb (1 μg/ml).

† 10 μg/ml.

‡ b.7.6 mAb (5 μg/ml).

proliferative response of neonatal B cells is inhibited by co-culture with anti-μ or anti-κ antibodies whilst the responses of adult splenic B cells are unaffected[19,20] (L.T., unpublished observations). It was therefore of interest to examine the effect of CD40L on neonatal B cells. To achieve this, spleen cells from 7-day-old mice were enriched for B cells and the proliferative responses to LPS of the resulting cells was assessed when cultured with or without anti-μ antibody in the presence or absence of CD40L. Anti-μ antibody was used for these experiments to avoid co-ligation of sIgM and sIgD. As expected, culture with anti-immunoglobulin antibody inhibited the LPS response of neonatal cells by >60% (Fig. 7). This inhibition of proliferation was, however, unaffected by the addition of CD40L–CD8 fusion protein even in the presence of cross-linking anti-CD8 mAb.

The effects of separate and co-ligation of CD40 and sIg on neonatal and mature, adult B cells were also examined. The findings (Table 1) confirmed that CD40 ligation induced proliferation in both adult and neonatal B cells, and addition of anti-μ to CD40L-stimulated adult cells induced a synergistic response. In contrast, the response of neonatal B cells to CD40 ligation was markedly inhibited by anti-μ and co-ligation of CD40 failed to prevent the anti-μ-induced inhibition of the response of neonatal B cells to LPS. The failure of CD40L to prevent anti-immunoglobulin-mediated inhibition of LPS responses by normal immature B cells could not result from a failure of the CD40L : CD40 interaction since the cross-linked fusion protein induced modest proliferation by itself (Fig. 7, Table 1) which was strongly enhanced in the presence of LPS (Table 1) and IL-4.[45] CD40 ligation could also be shown to induce class II upregulation on the neonatal B cells[45] as has been observed by others.[46]

DISCUSSION

It has long been established that immature B cells derived from either neonatal spleen or adult bone marrow are exquisitely sensitive to tolerance induction[16,17,32] and that this susceptibility is modified in the presence of T cells or their products. It thus seems likely that contact-mediated signals delivered by Th cells to B cells play a pivotal role in determining whether B cells display a negative or positive signalling pattern when their antigen receptors are ligated. To examine this possibility we have studied the effects of T cells and T-cell derived molecules on the responses of growth inhibitable B-cell lymphomas of immature phenotype and on normal immature B cells from neonatal spleen.

We have shown that the growth inhibitory effect of sIg ligation on cells of the WEHI 231 line is partially abrogated in the presence of mitogen-activated but not resting T cells. T cells activated by exposure to anti-CD3 antibody were, in contrast, unable to protect WEHI 231 cells from anti-immunoglobulin-induced growth arrest, suggesting that expression of CD40L, which has been shown to play a key role in T : B interactions and protects germinal centre B cells from apoptosis, may be important for the protective effect since this mode of activation only weakly induces expression of this molecule. Several reports have indicated that negative signalling in normal and transformed cells can be modulated by cytokines.[20,32,47] To test this possibility we investigated the effects of cell-free supernatants from two T-cell lines on anti-immunoglobulin-mediated growth inhibition of WEHI 231 cells. Supernatants from the IL-4-producing CBOVA line were effective in ameliorating growth inhibition whilst those of the BA5 line (which does not secrete IL-4) were not, suggesting a possible role for IL-4. However, we were unable to show any protective effect of this cytokine in experiments using rIL-4. These findings are similar to those reported by Tsubata & Honjo,[34] who also found that supernatants from some, but not all, T-cell lines and T hybridomas were able to rescue WEHI 231 cells from anti-immunoglobulin-induced cell death, although IL-1, IL-2, IL-4, IL-5 and IL-6, either alone or in combination, had no effect. In fact in one report where IL-4 was shown to counteract anti-immunoglobulin-mediated growth inhibition,[33] its effect alone was slight and the authors themselves speculated

that their data indicated a role for a novel T-cell-derived molecule.

Since ligation of CD40 had been shown to provide a survival signal for germinal centre B cells[48] and activated T-helper lines have been shown to secrete CD40L,[49] it was considered possible that this molecule may be active in our experiments. This was examined directly by using a soluble CD40L–CD8 fusion protein secreted by transfected J558 cells. Our experiments show that this molecule alone was sufficient to almost completely abrogate anti-immunoglobulin-induced growth inhibition in both WEHI 231 and CH33 cell lines, provided that the CD40L–CD8 construct was cross-linked with anti-CD8. Furthermore, the effects of CD40L on WEHI 231 cells could clearly be shown to involve the rescue of cells from apoptosis as judged by a dramatic reduction in DNA fragmentation following sIg ligation. These findings represent the first demonstration that ligation of B-cell CD40 by its soluble, natural ligand is, by itself, sufficient to rescue B cells from negative signalling via sIg. They also indicate that the intracellular signalling pathways underlying this rescue are more effectively engaged when CD40 is extensively cross-linked. Our results are in agreement with those reported by other workers who showed a similar protective effect on receptor-induced apoptosis in the human B-cell line Ramos when CD40 was ligated with an anti-CD40 mAb.[31] Co-culture of WEHI 231 cells with X63Ag8.653 myeloma cells transfected with an expression vector for murine CD40L has also been shown to rescue the cells from anti-immunoglobulin-induced growth arrest and incubation of WEHI 231 cells transfected with an expression vector for human CD40 with anti-human CD40 prevented anti-immunoglobulin-induced apoptosis.[34] More recently, ligation of CD40 with antibodies to murine CD40 has been shown to reverse anti-immunoglobulin-mediated growth arrest in WEHI 231 cells[50,51] by a mechanism involving the induction of the cell survival protein bcl-x_L.[51]

The susceptibility of WEHI 231 and CH33 cells to receptor-mediated growth arrest and apoptosis, together with the apparently immature phenotype of these lines, has led to their widespread adoption as models for clonal deletion or tolerance in immature B cells. However, when we examined the potential role of T-cell-derived signals on negative signalling in normal immature B cells from neonatal spleen, we found that CD40L was ineffective in reversing the anti-immunoglobulin-mediated inhibition of their responsiveness to the polyclonal mitogen LPS. This lack of effect could not be attributed to a lack of CD40 on the B cells since CD40 expression on neonatal B cells is similar to that on mature B cells from adult spleen[46] (our unpublished observations). Neither does it result from a lack of responsiveness to CD40 ligation in the neonatal B cells since they showed a modest degree of proliferation when cultured with CD40L–CD8 under cross-linking conditions, and have also been shown to upregulate their expression of class II MHC molecules and CD23 following ligation of CD40 with soluble CD40L.[45]

Interestingly, the proliferative response to CD40L-CD8 made by neonatal B cells was itself inhibited in cultures containing anti-immunoglobulin antibody, in marked contrast to the responses of mature B cells where CD40 and sIg ligation synergize to induce enhanced proliferation[35] (Table 1). Very similar observations have been made by others using mouse erythroleukaemia (MEL) cells transfected with a vector encoding murine CD40L to effect CD40 ligation.[52]

Taken together our data thus suggest that the mechanisms involved in the induction of growth arrest and apoptosis in immature B-lymphoma lines such as WEHI 231 and CH33 are distinguishable from those responsible for negative signalling in normal immature B cells. Several observations support this distinction. First, immature B cells have the phenotype sIgM positive, sIgD negative yet CH33 cells have been shown to express sIgD[53] and careful studies have shown that WEHI 231 cells also express high levels of a novel form of this isotype.[54] Second, the signals generated following sIg ligation in WEHI 231 cells have been reported to be qualitatively different from those generated in normal immature B cells.[55] When considered with the findings reported here these observations bring into question the usefulness of these lymphoma lines as models for tolerance induction.

Recent experiments have indicated that ligation of CD40 with mAb can rescue mature B cells from apoptosis induced by hypercross-linking of sIgM or sIgD.[56] In contrast to mature B cells, immature B cells can be induced to undergo apoptosis in response to soluble anti-immunoglobulin antibodies.[26] It seems likely that this mechanism underlies the inhibitory effects of anti-immunoglobulin antibodies on neonatal LPS responses. This suggests that it is important to examine the ability of CD40L to rescue neonatal splenic B cells from receptor-mediated apoptosis. These experiments are in progress.

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